Mechanisms involved in the cytotoxic and cytoprotective actions of saturated versus monounsaturated long-chain fatty acids in pancreatic β-cells

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Abstract

Long-chain saturated and monounsaturated fatty acids differ in their propensity to induce β-cell death *in vitro* with palmitate (C16:0) being cytotoxic, whereas palmitoleate (C16:1n-7) is cytoprotective. We now show that this cytoprotective capacity extends to a poorly metabolised C16:1n-7 derivative, methyl-palmitoleate (0·25 mM palmitate alone: 92±4% death after 18 h; palmitate plus 0·25 mM methyl-palmitoleate: 12±2%; $P<0·001$). Palmitoleate and its methylated derivative also acted as mitogens in cultured β-cells (5-bromo-2-deoxyuridine incorporation – control: 0·15±0·01 units; 0·25 mM palmitoleate: 0·22±0·01 units; $P<0·05$). It has been proposed that alterations in neutral lipid synthesis (particularly triacylglycerol (TAG) formation) might mediate the differential responses to saturated and unsaturated fatty acids and we have examined this proposition. Palmitate and palmitoleate both promoted β-cell phospholipid remodelling and increased TAG formation (control: 0·9±0·1 nmol TAG/10^6 cells; 0·25 mM palmitate: 1·55±0·07; 0·25 mM palmitoleate: 1·4±0·05; palmitate plus palmitoleate: 2·3±0·1). By contrast, methyl-palmitoleate failed to influence TAG levels (0·25 mM methyl-palmitoleate alone: 0·95±0·06 nmol TAG/10^6 cells; methyl-palmitoleate plus palmitate: 1·5±0·05) or its fatty acid composition in β-cells exposed to palmitate. The results suggest that monounsaturated fatty acids can promote cell viability and mitogenesis by a mechanism that does not require their metabolism and is independent of alterations in TAG formation.
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Introduction

The prevalence of type 2 diabetes is rising rapidly in many regions of the world, driven, in part, by changing lifestyles and dietary habits. In particular, it appears to be associated with increases in obesity, leading to alterations in the circulating lipid profile of affected individuals (Rhodes 2005, Kahn *et al.* 2006). This is significant since the disease is characterised by impaired insulin secretion and loss of insulin sensitivity followed by a progressive decline in β-cell mass, all of which may be influenced by alterations in lipid availability (Shimabukuro *et al.* 1998*b*, Ahren 2005, Marchetti *et al.* 2006, Newsholme *et al.* 2007).

Acute exposure of β-cells to free fatty acids (FFA) *in vitro* leads to enhanced rates of insulin secretion (Sako & Grill 1990, Newsholme *et al.* 2007), whereas more prolonged treatment can be associated with toxicity (Lupi *et al.* 2002, El-Assaad *et al.* 2003, Haber *et al.* 2003, 2006, Rhodes 2005, Azevedo-Martins *et al.* 2006). However, the latter occurs more dramatically during exposure to long-chain saturated FFA (e.g. palmitate (C16:0) or stearate (C18:0)) than in response to either shorter chain saturated (C14 or less) or longer chain unsaturated FFA (such as palmitoleate (C16:1n-7) or oleate (C18:1n-9)). Indeed, there is increasing evidence that when used within the physiological range of free concentrations, the latter species are directly cytoprotective to β-cells (Eitel *et al.* 2002, Maedler *et al.* 2003, Welters *et al.* 2004, 2006) although, when present in sufficiently high free concentrations, all fatty acids are likely to exert detrimental effects (Azevedo-Martins *et al.* 2006).

Since long-chain saturated and long-chain monounsaturated FFA can exert differential effects on β-cell viability, this implies that they must also elicit different intracellular responses in order to mediate these effects. Such differences have proved elusive to define but changes in neutral lipid accumulation could be involved since β-cells can store excess FFA in the form of triacylglycerol (TAG; Briaud *et al.* 2001, Unger & Zhou 2001, Unger & Orci 2002, Moffitt *et al.* 2005). However, the mechanistic consequences of this response have become the source of considerable debate with some investigators, proposing that diversion of FFA into intracellular TAG serves a cytoprotective function (Cnop *et al.* 2001, Listenberger *et al.* 2003), while others (e.g. Briaud *et al.* 2001, Moffitt *et al.* 2005) have seen this as a potentially toxic response.

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In the present project, we have studied the responses of pancreatic β-cells to incubation with extracellular FFA *in vitro* in more detail. We have employed a range of modified FFA species, both saturated and unsaturated, and have investigated whether the propensity of these to regulate cell viability correlates with their potential for intracellular metabolism to TAG.

Materials and Methods

Cell culture

The rat pancreatic β-cell line BRIN-BD11 was used in all experiments. The cells were cultured in RPMI-1640 medium (Invitrogen) containing 11 mM glucose supplemented with 10% fetal calf serum (PAA Laboratories, Yeovil, Somerset, UK), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). The cells were grown in 75 cm^2 flasks at 37 °C and 5% CO_2.

Treatment of cells with fatty acids

Stock solutions of 50 mM palmitate (Sigma) or 2-bromopalmitate (Avocado research chemicals, Morecambe, Lancs, UK) were prepared in 50% ethanol by heating to 70 °C. Palmitoleate (MP Biomedicals, Cambridge, UK) and methyl-palmitoleate (Sigma) were prepared by mixing with 90% ethanol at room temperature to produce stock solutions of 90 mM. The fatty acid preparations were then bound to 10% fatty acid-free BSA (MP Biomedicals) by incubation for 1 h at 37 °C. The mixture was added to RPMI-1640 medium (containing 11 mM glucose) deprived of fetal calf serum. The final concentrations present in the cell environment were 1% for BSA and 0·5% for ethanol. Cells were seeded into six-well plates at densities of 1×10^5 cells/well and incubated for 24 h in complete RPMI-1640 medium. The medium was then removed and replaced with relevant fatty acid/BSA complexes (in RPMI-1640 devoid of foetal calf serum) for a further 18 h. Controls received BSA and vehicle only.

Vital dye staining for estimation of viability

Following incubation, floating and attached cells were collected, centrifuged at 200 ***g*** for 5 min and resuspended in 250 μl complete RPMI-1640 plus 250 μl Trypan blue (0·4% in PBS). Viable and dead cells were counted using a haemocytometer and the number of dead cells expressed as a percentage of the total for each condition.

Fluorescence microscopic assays of cell viability

For the assessment of viable, necrotic and apoptotic cells, BRIN-BD11 cells previously treated with fatty acids were incubated for 5 min with a mixture of 50 μg/ml Hoechst 33342 (Sigma) and 5 μg/ml propidium iodide (Sigma) dissolved in PBS. Hoechst 33342 penetrates the plasma membrane of all cells, staining the DNA blue. Viable cells appeared pale blue, while apoptotic cells showed strong dark blue fluorescence due to DNA condensation. Propidium iodide can penetrate the cells only when their plasma membrane is damaged, leading to fluorescent pink staining of the nucleus. Consequently, necrotic cells were identified by their fluorescent pink colour. The stained cells were examined by fluorescence microscopy (Jankowska *et al.* 1997). The percentages of viable, necrotic and apoptotic cells were calculated after counting at least 100 cells for each experimental condition.

5-Bromo-2-deoxyuridine (BrdU) cell proliferation assay

Incorporation of BrdU, as an indication of cell proliferation, was measured with a commercial kit (Amersham). BRIN-BD11 cells were initially treated with fatty acids and then seeded into 96-well plates at a density of 5000 cells/well for 24 h. BrdU label was added to the culture medium during either 4 or 20 h of incubation, after which the cells were fixed and BrdU incorporation was assessed according to the manufacturer's instructions.

Analysis of phospholipid fatty acid composition and TAG formation

Gas chromatography was used for assessment of the fatty acid profile of β-cell phospholipids. BRIN-BD11 cells were seeded into 25 cm^2 flasks at a density of 1×10^6 cells/flask and were then exposed to fatty acids. The cells were trypsinised and centrifuged (200 ***g***; 5 min). Lipids were extracted by using a mixture of chloroform:methanol (2:1, v/v) containing butylated hydroxy-toluene (50 mg/l) as an antioxidant (Folch *et al.* 1957). Phospholipids were separated from other lipid classes by solid phase extraction using methods described elsewhere (Burdge *et al.* 2000) and were then saponified and methylated in methanol containing 2% (v/v) H_2SO_4 at 50 °C for 2 h. Fatty acid methyl esters (FAME) were recovered by extraction with hexane. FAME separation and analysis were carried out using a Hewlett Parckard 6890 gas chromatograph (Agilent Technologies, Wokingham, Berks, UK) with a BPX-70 capillary column (30 m length, 220 μm internal diameter, 0·25 μm film thickness, SGE Europe Limited, Milton Keynes, Bucks, UK). The gas chromatograph oven was programmed to hold for 2 min at the starting temperature of +115 °C then increased at +10 °C/min to 200 °C and hold there for 10 min and finally raised at +60 °C/min to 245 °C and hold for 4 min at the temperature of 245 °C. FAME were identified by comparison of peak retention times to those of known standards. Chemstation software was used for peak area quantification. The quantity of fatty acids was calculated using appropriate internal standards.

Triglyceride (TG) levels of BRIN-BD11 cells were measured with a commercial kit designed for analysis of cellular triglyceride content (Zen-bio Triglyceride Assay Kit # TG-1-NC). Cells were seeded into 24-well plates at densities of 1×10^5cells/well and treated with the appropriate test reagents for 18 h. They were then lysed and processed

according to the manufacturer's instructions in parallel with standard glycerol solutions provided in the kit. TG levels were estimated from the release of glycerol in experimental samples and were measured spectrophotometrically at 540 nm.

Insulin secretion

BRIN-BD11 cells were seeded into 24-well plates at a density of 5×10^4 cells/well and incubated for 18 h in the absence or presence of fatty acids, as appropriate. The medium was then removed and the cells washed and incubated in a bicarbonate-buffered physiological saline solution (Gey & Gey 1936) containing 4 mM glucose and 1 mg/ml BSA for 1 h. This buffer was then replaced with fresh incubation buffer containing test reagents and insulin secretion measured by RIA after incubation at 37 °C for 1 h.

Data analysis

All experiments were performed on at least three separate occasions and triplicates of each condition were used in each experiment. The results are expressed as mean ± S.E.M. and the level of significance was calculated by using Student's *t*-test or ANOVA and was regarded as significant when $P<0{\cdot}05$.

Results

Effects of fatty acids on β-cell viability

In confirmation of previous observations (Welters *et al.* 2004, 2006), incubation of BRIN-BD11 cells with palmitate dose-dependently reduced their viability as judged by either trypan blue exclusion or staining with propidium iodide/Hoechst 33342. This effect was primarily due to increased apoptosis as judged by fluorescence microscopy after staining with propidium iodide and Hoechst 33342 (Fig. 1). By contrast, palmitoleate was not toxic to the cells at concentrations up to 0.25 mM. Cells exposed to both palmitate and palmitoleate simultaneously also retained viability (0·25 mM palmitate alone: 89 ± 8% non-viable cells; 0·25 mM palmitoleate alone: 9 ± 3% non-viable cells; palmitate plus palmitoleate: 19 ± 3% non-viable cells ($P<0{\cdot}001$ versus palmitate alone)), suggesting that palmitoleate was actively protective against palmitate-induced cytotoxicity.

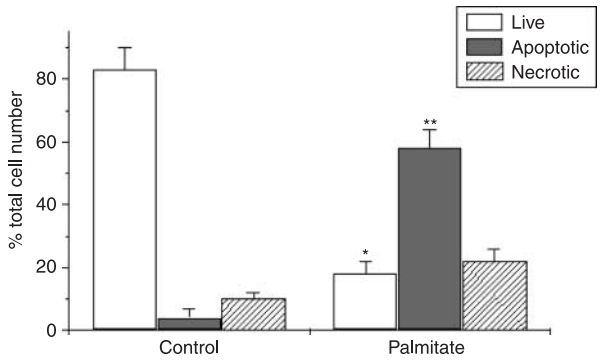

Figure 1 Mode of β-cell death following exposure to palmitate. BRIN-BD11 cells were treated for 18 h in the absence (control) or presence of 0·1 mM palmitate. The numbers of live (white bars), apoptotic (solid grey bars) and necrotic (cross-hatched bars) cells were estimated by fluorescence microscopy after staining with propidium iodide and Hoechst 33342. *Significantly reduced ($P<0{\cdot}001$) when compared with vehicle-treated cells; **significantly increased ($P<0{\cdot}001$) when compared with vehicle-treated cells.

Examination of the cytoprotective effect of palmitoleate at the level of β-cell growth

It was important to establish whether the protective actions of palmitoleate result in true preservation of β-cell viability or whether this fatty acid simply delays the onset of apoptosis beyond the time course of the present experiments. A variety of methods were employed to determine this and, in particular, studies were undertaken to examine β-cell growth and proliferation following exposure to different fatty acids. Initially, BRIN-BD11 cells were treated with 0·25 mM palmitate alone, 0·25 mM palmitoleate alone or a combination of the two fatty acids for 18 h, after which time the fatty acids were removed and the culture medium replaced. Cell growth was then monitored over the ensuing period (Fig. 2). The results revealed that 18-h exposure to palmitate led to a marked loss of viability (up to 80%) and, as expected, these cells failed to proliferate during subsequent incubation. By contrast, those cells that had been treated with palmitoleate alone or with palmitoleate in the presence of palmitate exhibited an increased rate of proliferation over the period following initial fatty acid treatment. Thus, the protection afforded by palmitoleate extended not only to maintaining cell viability but also to facilitating cell

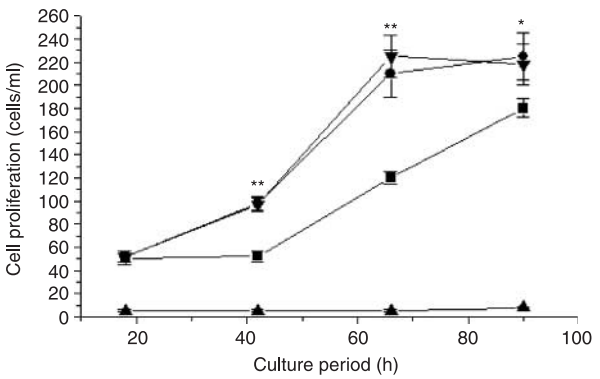

Figure 2 Effects of fatty acids on β-cell proliferation. BRIN-BD11 cells were initially incubated either in the absence of fatty acids (squares) or in the presence of 0·25 mM palmitate alone (triangles), 0·25 mM palmitoleate alone (circles) or the combination of 0·25 mM palmitate plus 0·25 mM palmitoleate (inverted triangles) for 18 h. After this time, the fatty acids were removed, the culture medium replaced and cell growth monitored over the subsequent 72 h. *$P<0{\cdot}05$ when compared with untreated cells; **$P<0{\cdot}01$ when compared with untreated cells.

proliferation. Indeed, the rate of proliferation seen in cells exposed to either palmitoleate alone or palmitoleate plus palmitate was significantly higher than that measured in cells treated with vehicle alone (Fig. 2).

Further confirmation that palmitoleate treatment results in enhanced cell growth was achieved by measuring β-cell DNA synthesis more directly following fatty acid exposure (Fig. 3). To achieve this, BRIN-BD11 cells were exposed to BrdU following the relevant culture period with fatty acids. Cells treated with palmitate alone showed reduced proliferation when compared with control (as evidenced by attenuated incorporation of BrdU), whereas those treated with palmitoleate (either alone or in the combined presence of palmitate) demonstrated the highest levels of DNA synthesis (Fig. 3). Indeed, the rate of DNA synthesis measured under these conditions surpassed that of cells grown in complete medium throughout (complete medium: $0{\cdot}18 \pm 0{\cdot}01$ A_{450} units; palmitoleate: $0{\cdot}22 \pm 0{\cdot}01$ units; $P < 0{\cdot}01$).

The cytoprotection afforded by palmitoleate over an 18-h incubation period also extended to the preservation of the insulin secretory function of the cells (Fig. 4). Insulin secretion from cells exposed to palmitate alone could not be tested due to the extensive loss of viability under these conditions. However, cells exposed to palmitate in the presence of palmitoleate (where viability was maintained) responded to a nutrient stimulus, α-ketoisocaproate, with an equivalent response to that seen in untreated controls (Fig. 4) or those exposed to palmitoleate alone. Thus, at least during 18 h of fatty acid exposure, insulin secretion was not compromised when the monounsaturate was present.

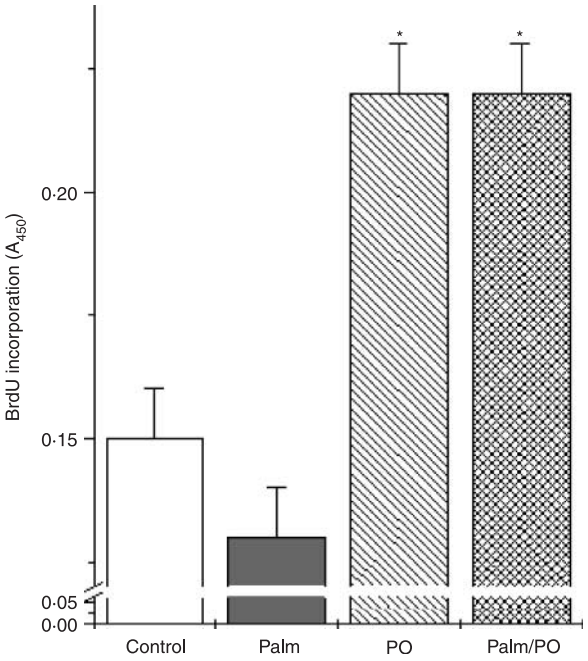

Figure 3 Measurement of DNA synthesis in cells exposed to fatty acids. BRIN-BD11 cells were cultured with vehicle (white bars) 0·25 mM palmitate (solid grey bars), 0·25 mM palmitoleate (hatched bars) or 0·25 mM palmitate plus 0·25 mM palmitoleate (cross-hatched bars) for 18 h. The level of cell proliferation was estimated via the incorporation of BrdU during the incubation period. *$P < 0{\cdot}01$ when compared with untreated cells.

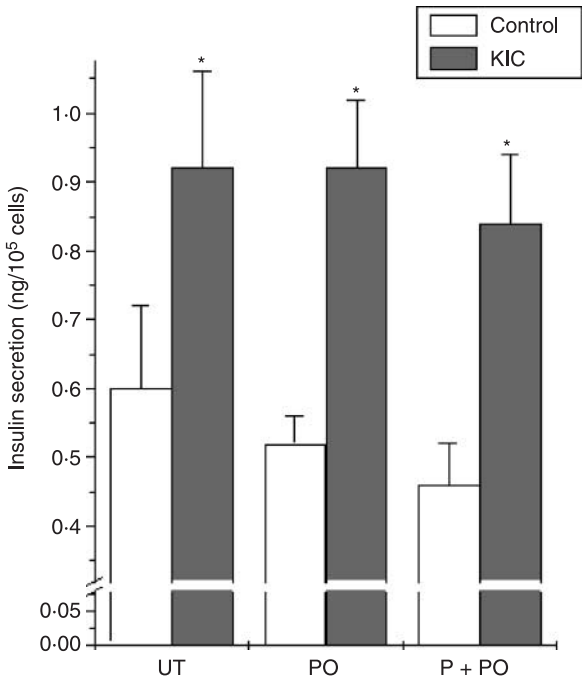

Figure 4 Insulin secretory responses of BRIN-BD11 cells following exposure to fatty acids. Cells were cultured for 18 h either without fatty acids (UT) or in the presence of 0·25 mM palmitoleate alone (PO) or 0·25 mM palmitoleate plus 0·25 mM palmitate (P+PO). They were then washed and incubated in buffer containing 4 mM glucose alone (control; white bars) or 4 mM glucose plus 10 mM α-ketoisocaproate (KIC; grey bars) for 1 h. After this time, the medium was sampled and insulin was measured by RIA. *$P < 0{\cdot}01$ relative to the absence of KIC.

Measurement of the lipid content of β-cells cultured in the presence of fatty acids

It has been proposed that the behaviour of β-cells following fatty acid exposure is determined by alterations in lipid disposition, but this area remains controversial since both protective and detrimental affects have been attributed to altered lipid handling. Therefore, to evaluate the effects of fatty acids on lipid formation in cultured β-cells, lipids were extracted from BRIN-BD11 cells and their fatty acid composition analysed by gas chromatography. Initial experiments were undertaken to establish the total fatty acid composition of the neutral and phospholipid fractions within BRIN-BD11 cells incubated under control conditions. The results demonstrated that the most abundant fatty acid species were palmitate (C16:0), oleate (C18:1n-9), stearate (C18:0) and linoleate (C18:2n-6) with lower, but measurable amounts of palmitoleate (C16:1n-7) and arachidonate (C20:4n-6; Fig. 5).

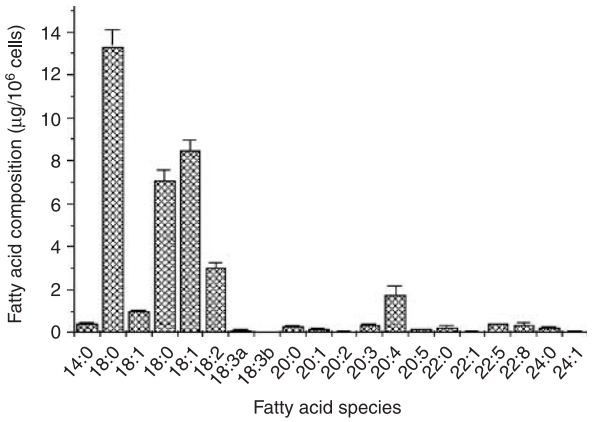

Figure 5 Fatty acid species present in untreated BRIN-BD11 cells. BRIN-BD11 cells were grown in complete medium and their total fatty acid composition analysed by gas chromatography after lipid extraction.

Treatment of the cells with exogenous fatty acids resulted in an alteration in their phospholipid fatty acid profile, without any change in the total phospholipid content. The total content of fatty acids present in phospholipids was ~120 μg/10^6 cells, suggesting that this pool of fatty acid (FA) is large with respect to the total that is esterified within TAG (see below). As expected, the alterations in the phospholipids FA profile mirrored the relative availability of each fatty acid in the culture medium (Fig. 6). Thus, palmitate-treated cells displayed an elevation of palmitate in the phospholipid fraction, consistent with increased incorporation of this fatty acid under these conditions. This occurred primarily at the expense of oleate and stearate. By contrast, palmitoleate-treated cells displayed an elevation of this fatty acid in their phospholipids with a concomitant reduction in palmitate, stearate and oleate. Cells exposed to both palmitate and palmitoleate showed an intermediate increase in the

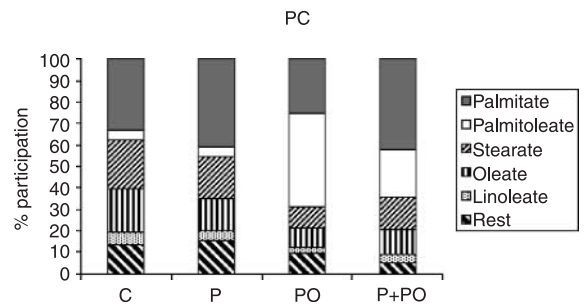

Figure 6 Fatty acid composition of the phosphatidylcholine fraction of BRIN-BD11 cells following incubation with exogenous fatty acids. BRIN-BD11 cells were treated with vehicle (C) 0·25 mM palmitate (P) 0·25 mM palmitoleate (PO) or 0·25 mM palmitate plus 0·25 mM palmitoleate (P+PO) for 18 h. Phosphatidylcholine (PC) was extracted as a representative phospholipid and quantified by gas chromatography. Results are presented as the percentage of each fatty acid measured within the PC fraction under the relevant experimental conditions.

incorporation of each of these species within their phospholipids.

In addition to altering the phospholipid fatty acid composition of BRIN-BD11 cells, exposure to elevated concentrations of exogenous fatty acids also affected their TAG content (Fig. 7a). The total amount of FA contained within the TAG fraction amounted to ~25 μg/10^6 cells and, as such, this represents a smaller pool than that held in phospholipids. Nevertheless, addition of either 0·25 mM palmitate or 0·25 mM palmitoleate to the culture medium significantly raised the total TAG content of BRIN-BD11 cells by comparison with control cells. Culture of cells in the simultaneous presence of palmitate and palmitoleate for 18 h resulted in a further rise in TAG, such that the net effect was additive (Fig. 7a).

To investigate these effects further and to discover whether altered TAG levels show any correlation with the changes in viability observed in response to different fatty acids, the effects of a range of additional conditions were also examined. Initially, 2-bromopalmitate (2-BP), an analogue of palmitate that can be thioesterified to Coenzyme-A but cannot then be further metabolised, was studied. The 2-BP is expected to reduce the total Coenzyme-A pool by sequestration (Chase & Tubbs 1972, Oakes *et al*. 1999) and thereby to cause a reduction in TAG synthesis from exogenous palmitate. In support of this, TAG formation was inhibited in cells exposed to palmitate plus 2-BP when compared with cells exposed to palmitate alone (Fig. 7*a*). This result confirms that β-cell TAG formation is influenced by the availability of FFA for esterification.

Next, methyl-palmitoleate, an analogue of palmitoleate that cannot be esterified to Co-enzyme A, was employed. As expected, exposure of cells to methyl-palmitoleate alone, failed to influence their TAG content (Fig. 7*a*). More significantly, the co-presence of this fatty acid failed to alter the TAG content of palmitate-treated cells, suggesting that it did not alter the metabolic routing of palmitate into TAG (Fig. 7*a*). To confirm this result, additional studies were conducted in which the fatty acid composition of BRIN-BD11 cell TAG was analysed after incubation of cells with palmitate and methyl-palmitoleate. As predicted, the increased incorporation of palmitate into TAG seen under these conditions was not attenuated by the presence of methyl-palmitoleate (control cells: 1·77±0·04 μg palmitate in TAG/10^6 cells; 0·25 mM palmitate alone: 2·57±0·28 μg ($P<0·01$); 0·25 mM methyl-palmitoleate alone: 1·02±0·14 μg; palmitate plus methyl-palmitoleate: 2·39±0·11 μg (not significantly different from palmitate alone)). Despite this, methyl-palmitoleate abolished the cytotoxicity induced by palmitate as revealed by cell viability assays (Fig. 7*b*). Indeed, its protective actions were equipotent with palmitoleate (Fig. 8) and, like palmitoleate, it also promoted β-cell growth following co-incubation with palmitate (not presented). Thus, the cytoprotective and growth-promoting properties of methyl-palmitoleate and palmitoleate were

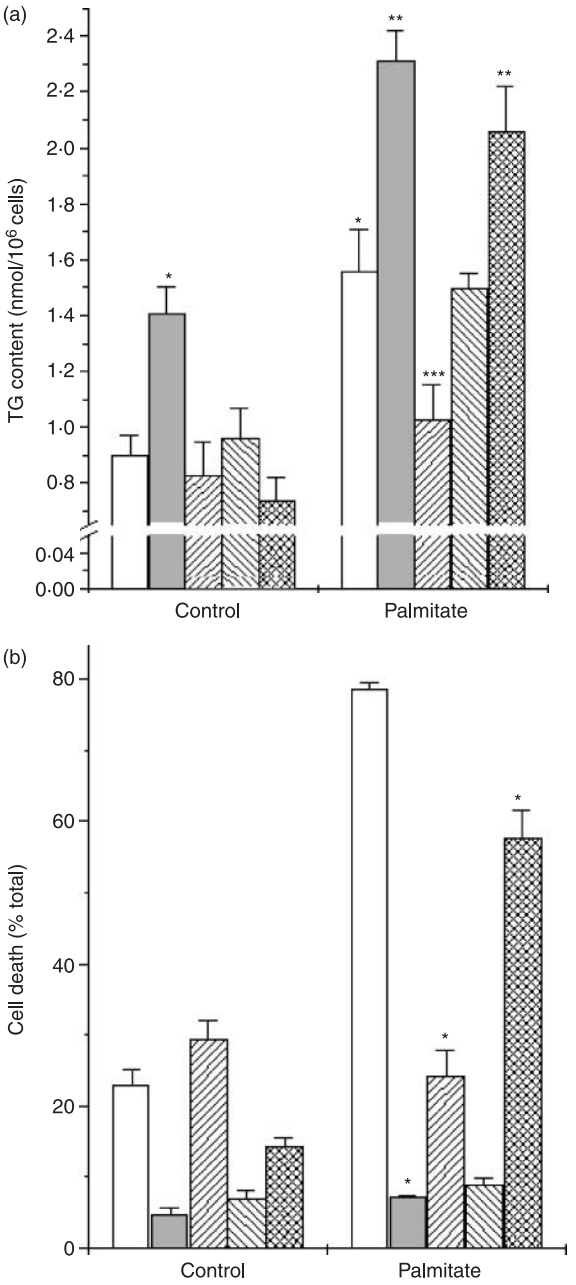

Figure 7 Triglyceride (TG) content (a) and cell viability (b) of fatty acid treated β-cells. BRIN-BD11 cells were treated for 18 h with vehicle (white bars) 0·25 mM palmitoleate (solid grey bars), 0·25 mM 2-bromopalmitate (right-hatched bars) 0·25 mM methyl-palmitoleate (left-hatched bars) or 200 mg/ml etomoxir (cross-hatched bars) in the absence (control) or presence of 0·25 mM palmitate. The cells were then either lysed for analysis of intracellular triglyceride (a) or stained with trypan blue to assess their viability (b). (a)*$P<0·01$ when compared with vehicle-treated cells. **$P<0·01$ when compared with cells exposed to palmitate alone. ***$P<0·05$ when compared with cells exposed to palmitate alone. (b) *$P<0·01$ when compared with cells exposed to palmitate alone.

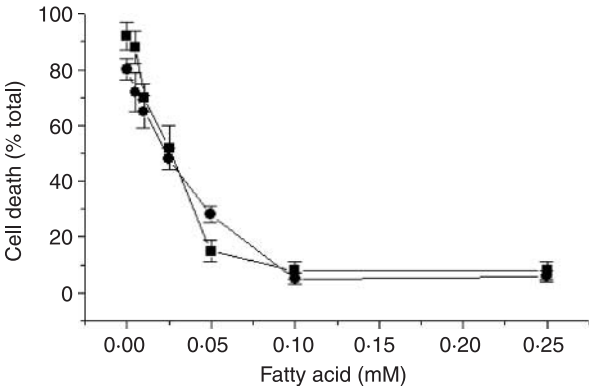

Figure 8 Dose-dependent inhibition of palmitate-induced β-cell death by palmitoleate and methyl-palmitoleate. BRIN-BD11 cells were incubated with increasing concentrations of palmitoleate (squares) or methyl-palmitoleate (circles) in the presence of 0·25 mM palmitate for 18 h. The extent of cell death was estimated after staining with trypan blue. Significant inhibition ($P<0·05$) of palmitate-induced cytotoxicity was achieved with concentrations of 0·025 mM or greater for both palmitoleate and methyl-palmitoleate.

equivalent even though these fatty acids exerted differential effects on the TAG content of palmitate-treated cells.

Etomoxir, an inhibitor of carnityl palmitoyltransferase-1 (CPT-1), attenuates the uptake of fatty acids into mitochondria thereby reducing the rate of β-oxidation (Chen *et al.* 1994) and promoting fatty acid availability for TAG synthesis. Accordingly, treatment of BRIN-BD11 cells with etomoxir in the presence of palmitate resulted in enhanced TAG formation by comparison with cells exposed to palmitate alone (Fig. 7a). However, viability assays revealed that the extent of cell death under these conditions was lower than that seen with palmitate alone (Fig. 7b).

Discussion

It has become increasingly clear that long-chain fatty acids exert complex, time-dependent effects on pancreatic β-cells, with short-term exposure leading to enhancement of insulin secretion, while chronic treatment is associated with attenuated insulin secretion and reduced viability (Zhou & Grill 1994, Milburn *et al.* 1995, Unger 1995, Bollheimer *et al.* 1998, Shimabukuro *et al.* 1998*b*, McGarry & Dobbins 1999). The latter has sometimes been called 'lipotoxicity' and could be taken as a relatively non-specific process arising from a generalised elevation in circulating fatty acid concentrations *in vivo*. Lipotoxicity can also be observed *in vitro* (Shimabukuro *et al.* 1998*b*, Cnop *et al.* 2001, Eitel *et al.* 2002, Lupi *et al.* 2002, Piro *et al.* 2002, Maestre *et al.* 2003, Welters *et al.* 2004, Moffitt *et al.* 2005, Azevedo-Martins *et al.* 2006) when pancreatic β-cells are incubated in the presence of exogenous fatty acids although it is clear that under these conditions, loss of β-cell viability occurs over a much shorter timescale than that seen *in vivo*. Indeed, since the combinations of fatty acids

used *in vitro* do not fully recapitulate the situation *in vivo*, the data must always be interpreted with caution. Nevertheless, it is accepted that *in vitro* models of lipotoxicity can provide important information about the underlying mechanisms involved (Newsholme *et al.* 2007).

In the present work, we have used an *in vitro* system to demonstrate that saturated and unsaturated fatty acids differ markedly in their ability to regulate β-cell viability. In particular, the results reveal that the long-chain saturated fatty acid palmitate readily promotes β-cell apoptosis, whereas the monounsaturated palmitoleate is potently cytoprotective. Thus, at least under *in vitro* conditions, the ability to induce β-cell lipotoxicity appears to be a feature that is characteristic mainly of long-chain saturated fatty acids (Eitel *et al.* 2002, El-Assaad *et al.* 2003, Maedler *et al.* 2003, Welters *et al.* 2004, 2006).

One area of specific controversy concerns the role played by intracellular TAG in mediating the cytotoxic actions of long-chain fatty acids. Some workers have considered intracellular TAG stores as potential mediators of lipotoxicity (Shimabukuro *et al.* 1998*a*,*b*) by virtue of their capacity to mediate the physical disruption of cells (Moffitt *et al.* 2005). Conversely, others have suggested that TAG represents a reservoir of intracellular lipid into which exogenous FFA can be sequestered as a means to minimise any cytotoxic potential (Cnop *et al.* 2001, Listenberger *et al.* 2003). This issue remains unresolved and, in the present studies, we have attempted to clarify the situation by monitoring β-cell TAG formation in parallel with alterations in viability when cells are exposed to saturated and unsaturated fatty acids either alone or in combination.

As expected, fatty acid treatment caused a net increase in total TAG formation in BRIN-BD11 cells. The magnitude of this increase was similar when cells were exposed to either palmitate or palmitoleate alone (0·25 mM) and, when the two fatty acids were combined, an additive increase in TAG formation was seen. These results confirm that the intracellular TAG pool plays an active role in sequestration of exogenous fatty acids during incubation of BRIN-BD11 β-cells *in vitro* (Dixon *et al.* 2004).

Moffitt *et al.* (2005) have argued that the species of TAG formed during fatty acid exposure of β-cells may be critical in determining the final outcome in terms of overall cell viability. In particular, they suggest that TAG species that contain predominantly palmitate may be particularly cytotoxic and that the apparent protective actions of monounsaturates could be due to their ability to alter the TAG composition. In order to address this issue, we employed a derivative of palmitoleate which is methylated on the carboxyl group and, as a consequence, cannot be incorporated into TAG. In support of this, incubation of BRIN-BD11 cells with 0·25 mM methyl-palmitoleate failed to generate an increase in TAG and it did not modify the extent of TAG formation seen in palmitate-treated cells. Moreover, this FA failed to attenuate the increase in palmitate incorporation into TAG when the two FAs were added in combination, suggesting that under these conditions, the TAG was still predominantly composed of tripalmitin.

Despite its failure to modify TAG levels in palmitate-treated cells, methyl-palmitoleate completely recapitulated the cytoprotective actions of its parent compound, palmitoleate, and it was equipotent with palmitoleate in this respect. These results suggest very strongly that the ability of the monounsaturates to promote β-cell viability in cells exposed to palmitate does not occur as a direct consequence of altered TAG formation or altered TAG composition. They also imply that the TAG formed in palmitate-treated cells is not directly cytotoxic since methyl-palmitoleate failed to modify TAG but maintained cell viability.

Additional support for these conclusions comes from the results of experiments performed with an inhibitor of CPT-1, etomoxir. CPT-1 catalyses the transit of fatty acids from the cytosol into the mitochondria where they are then available for metabolism by β-oxidation. Inhibition of CPT-1 therefore increases the availability of fatty acids in the cytosol, leading to their diversion away from oxidative metabolism and towards TAG formation. Accordingly, cells exposed to palmitate plus etomoxir contained more TAG than cells exposed to either compound alone. Moreover, since this increase in TAG occurred in response to the addition of exogenous palmitate, it is reasonable to deduce that it was relatively enriched in palmitate (i.e. that it was mainly tripalmitin). Despite this, the overall extent of cell viability was not further decreased under these conditions but, rather, etomoxir attenuated the loss of viability caused by palmitate.

Taken together, the data obtained with palmitoleate, methyl-palmitoleate and etomoxir reveal that changes in the formation of TAG from exogenous palmitate do not correlate with altered viability and they imply that TAG formation from palmitate is not directly cytotoxic under these conditions. Indeed, as proposed by Cnop *et al.* (2001), the results are not inconsistent with the possibility that TAG formation from palmitate can, under certain circumstances, be directly cytoprotective to β-cells (although this is unlikely to account completely for the protection afforded by monounsaturated fatty acids reported here).

In addition to causing alterations in TAG formation, exposure of β-cells to fatty acids also changed the relative composition of the cellular phospholipid pool, and this occurred in concert with the prevailing fatty acid availability. These observations imply that aspects of cell function that are determined largely by the physical properties of cellular membranes could vary in response to different exogenous fatty acids. Indeed, such effects may well contribute to the differential toxicity of saturated versus unsaturated molecules, perhaps by altering the activity of lipid-dependent enzymes such as phospholipases.

The results obtained in the current study also provide a number of additional insights into the actions of fatty acids in β-cells. Most importantly, they reveal that the effects of monounsaturates are not limited to the blockade of apoptosis but they show that as reported for breast cancer cells (Hardy

et al. 2005), these molecules can also directly promote the growth and proliferation of β-cells. The cytoprotection achieved by palmitoleate during an 18-h incubation period also preserved the insulin secretory responses of the cells.

The finding that the cytoprotective and mitogenic actions of palmitoleate were preserved when the carboxyl group of the fatty acid was methylated implies that these actions were not secondary to increased metabolism of palmitoleate. The present work has not disclosed the molecular mechanisms involved, but it is noteworthy that some actions of fatty acids in β-cells are now attributed to the activation of a subgroup of G-protein-coupled receptors (GPRs; Itoh *et al.* 2003, Kotarsky *et al.* 2003, Fujiwara *et al.* 2005, Hardy *et al.* 2005, Salehi *et al.* 2005, Steneberg *et al.* 2005, Gromada 2006, Schnell *et al.* 2007). One such molecule, GPR120, has recently been reported to mediate the anti-apoptotic effects of fatty acids in intestinal L-cells (Katsuma *et al.* 2005), but it remains to be determined whether GPR120 may be responsible for mediating the actions of monounsaturates observed in the present study.

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